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# **MANTLE CELL LYMPHOMA; STUDIES OF PREDICTIVE MARKERS, THE ROLE OF THE MICROENVIRONMENT IN DISEASE DEVELOPMENT AND IDENTIFICATION OF NEW POTENTIAL TARGETS FOR THERAPY**

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# Mantle cell lymphoma; studies of predictive markers, the role of the microenvironment in disease development and identification of new potential targets of therapy

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**Stockholm 2020**



*Till Siri, Flora & Bernard*



## ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive B cell lymphoma and accounts for 5-10 % of all non-Hodgkin lymphomas (NHL). In Sweden, the incidence of NHL is 21.6 and 15.3 per 100 000 persons for men and women, respectively, which result in almost 100-200 new MCL cases per year. MCL was considered an own entity in 1994 and is characterized by the gene translocation t(11;14)(q13;q32), *CCND1/IGH*, that occurs in pre-B cells and increases the expression of cyclin D1. Cyclin D1 drives the cell cycle thus promoting cell proliferation. This is believed to be the first oncogenic event in MCL. Additional oncogenic events are needed for MCL pathogenesis including aberrations in DNA-damage repair, cell cycle control and cell survival pathways. MCL is also dependent on the tissue microenvironment in order to survive, since MCL cells kept *in vitro* quickly die if not co-cultured with e.g. stromal cells.

The typical MCL patient is male and 65-70 years old at the time of diagnosis. The disease is often spread to bone marrow, blood, lymph nodes, Waldeyers ring, gastro intestinal tract and spleen. MCL usually respond well to first line treatment but then invariably relapses.

In my studies, I have used a well characterized population-based cohort of MCL patients from the Stockholm region. I have used this cohort to study various aspects of the disease, including potential markers to identify a more indolent disease course, the impact of the lymphoma tissue microenvironment and the cannabinoid receptors and the role of gender, comorbidities and choice of therapy in relation to outcome.

In paper I and paper IV we identify a subpopulation of MCL patients with a more indolent disease course, not needing therapy for at least two years after diagnosis. Several studies have tried to find a good marker predicting indolent disease, and the transcription factor SOX11 was initially suggested to carry such information. In paper I, we verify known prognostic markers such as age, elevated ECOG performance status, elevated lactate dehydrogenase (LDH) and high p53 expression, while SOX11 could not be used alone to predict disease course. In paper IV we found that women are older and more often have elevated LDH at the time of diagnosis. After introduction of the monoclonal CD20 antibody rituximab to first line therapy, the OS increased. In contrast to most studies we found that male gender was a positive prognostic factor. The median overall survival (OS) for all patients was 3,9 years and for those receiving or were intended for ASCT (27,3%) 16,3 years.

In paper II we report that the tissue microenvironment in MCL correlate to patient outcome. In MCL samples, T cells were fewer than in reactive lymph nodes and higher CD4+/CD8+ ratio correlated to longer OS.

In paper III we study the impact of the two cannabinoid receptors type 1 and type 2 (CB1 and CB2) which are upregulated in MCL cells compared to non-malignant B cells. We analysed CB1, CB2 and the enzymes involved in synthesis and metabolism of the cannabinoid receptor agonist anandamide (NAPEPLD and FAAH, respectively). All MCL had upregulated expression of NAPEPLD and most had low expression of FAAH compared to normal B cells thus favouring increased anandamide levels. Further, we found high expression of CB1 and high CB1 expression correlated to SOX11-positivity while low CB1 expression correlated to leucocytosis and lymphocytosis. High amount of FAAH also correlated to leucocytosis and lymphocytosis and to p53-positivity. We found no prediction

for outcome, but the endocannabinoid system is still a potential target for therapy that needs to be investigated further.



## LIST OF SCIENTIFIC PAPERS

- I. **Lina Nygren**, Stefanie Baumgartner-Wennerholm, Monica Klimkowska, Birger Christensson, Eva Kimby & Birgitta Sander.  
Prognostic role of SOX11 in a population-based cohort of mantle cell lymphoma. *Blood* 2012 May 3;119(18):4215-23.
- II. Agata M. Wasik, **Lina Nygren**, Stefan Almestrand, Fang Zong, Jenny Flygare, Stefanie Baumgartner-Wennerholm, Leonie Saft, Patrik Andersson, Eva Kimby, Björn E. Wahlin, Birger Christensson & Birgitta Sander.  
Perturbations in the endocannabinoid system in mantle cell lymphoma: correlations to clinical and pathological features. *Oncoscience* 2014 Sep 4;1(8):550-7.
- III. **Lina Nygren**, Agata M. Wasik, Stefanie Baumgartner-Wennerholm, Åsa Jeppsson-Ahlberg, Monica Klimkowska, Patrik Andersson, Daren Buhrkuhl, Birger Christensson, Eva Kimby, Björn E. Wahlin & Birgitta Sander.  
T cell levels are prognostic in mantle cell lymphoma. *Clinical Cancer Research* 2014 Dec 1;20(23):6096-104.
- IV. **Lina Nygren**, Agata M. Wasik, Birger Christensson, Björn E. Wahlin, Birgitta Sander & Eva Kimby.  
Mantle cell lymphoma, a population-based long-term follow-up study on patients in the Stockholm region: clinical and pathological presentation, course and treatment. *Manuscript*

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High-resolution chromatin immunoprecipitation (ChIP) sequencing reveals novel binding targets and prognostic role for SOX11 in mantle cell lymphoma. *Oncogene*. 2015 Mar 5;34(10):1231–40.

Mundt F, Merrien M, **Nygren L**, Sutton LA, Christensson B, Wahlin BE, Rosenquist R, Sander B, Wasik AM.

Expression of GNAZ, encoding the  $G\alpha_z$  protein, predicts survival in mantle cell lymphoma. *British Journal of Haematology*. 2019 May;185(4):708–712.

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## LIST OF ABBREVIATIONS

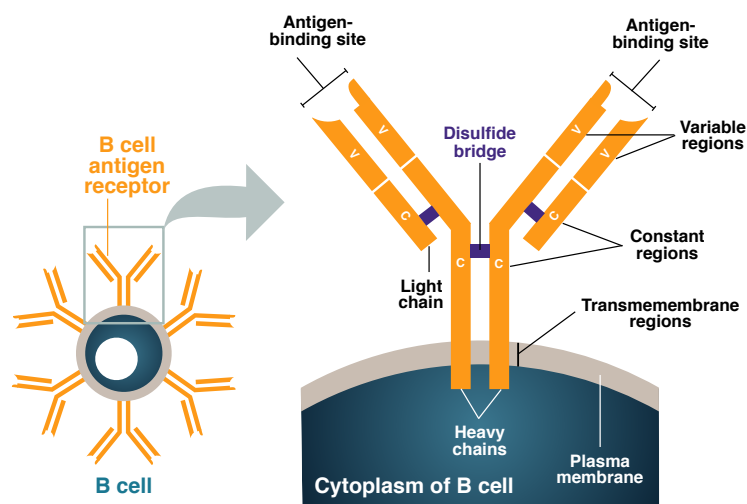
2-AG	2-arachidonoyl glyceryl
AEA	N-arachidonoyl ethanolamine (also called anandamide)
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
ASCT	Autologous stem cell transplantation
BCR	B cell receptor
BM	Bone marrow
BV	Blastoid variant
BTK	Bruton's tyrosine kinase
CAR-T cell	Chimeric antigen receptor-T cell
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CLL	Chronic lymphocytic leukaemia
D	Diffuse
DLBCL	Diffuse large B cell lymphoma
ECOG	Eastern Cooperative Oncology Group
ECS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
FISH	Fluorescent in situ hybridization
GI	Gastro intestinal
HLA	Human leucocyte antigen
ICOS	Inducible T cell co-stimulator
Ig	Immunoglobulin
IGH	Immunoglobulin heavy chain
IHC	Immunohistochemistry
IL	Interleukin
LAM	Lymphoma associated macrophages
LDH	Lactate dehydrogenase

LSS	Lymphoma specific survival
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MCL	Mantle cell lymphoma
MIPI	Mantle cell lymphoma International Prognostic Index
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stroma cell
MZ	Mantle zone
N	Nodular
NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next-generation sequencing
NHL	Non-Hodgkin lymphomas
NK cell	Natural killer cell
OS	Overall survival
PTLD	Posttransplant lymphoproliferative disorder
qPCR	Quantitative polymerase chain reaction
R	Rituximab
RT-PCR	Real-time polymerase chain reaction
SMH	Somatic hypermutations
SOX	Sex-determining region Y-box
SRY	Sex determining region on the Y chromosome
SYK	Spleen tyrosine kinase
THC	$\Delta^9$ -tetrahydrocannabinol
TLR	Toll-like receptor

# 1 B CELLS, INNATE AND ADAPTIVE IMMUNITY AND B CELL DEVELOPMENT

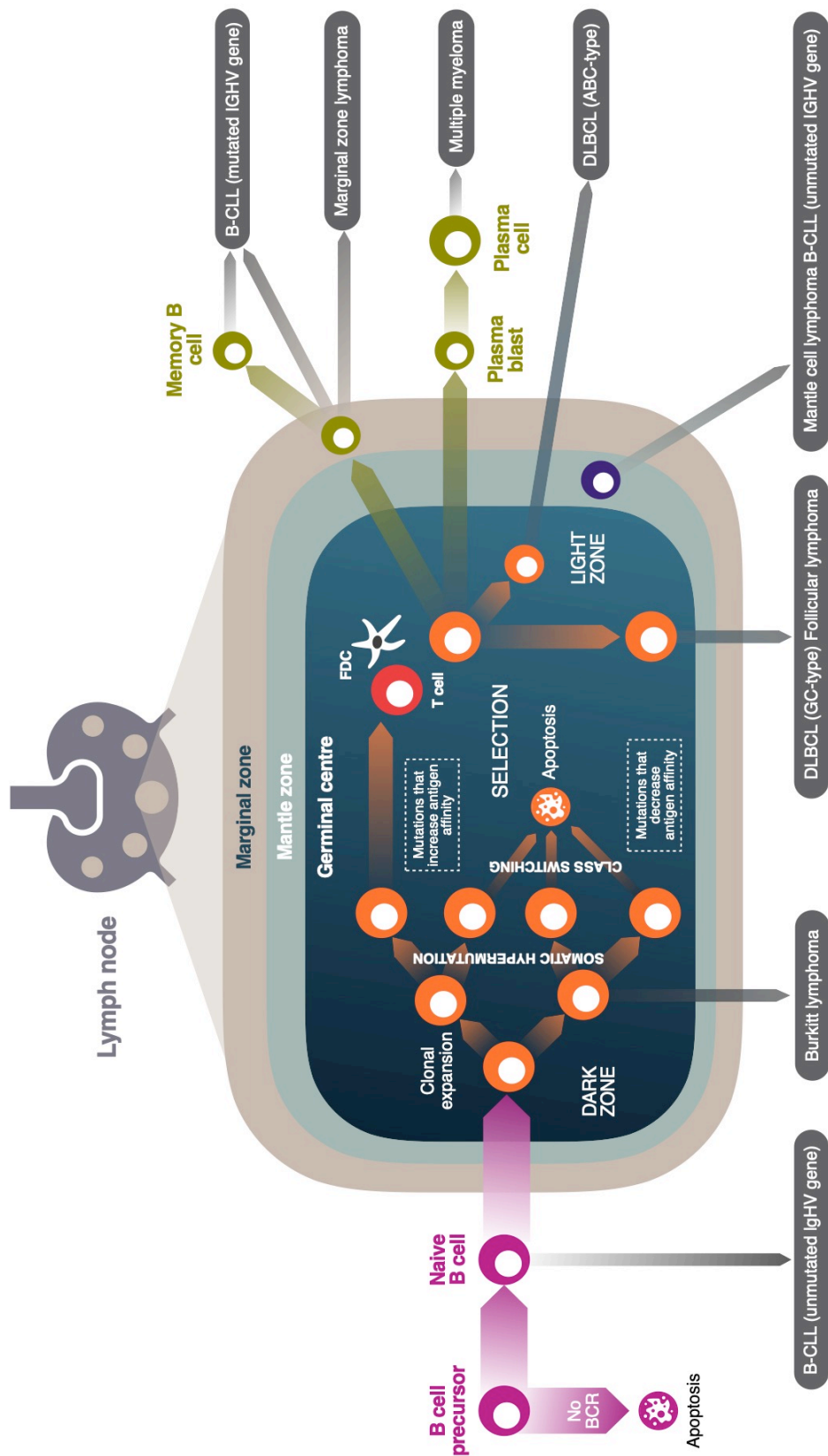
## 1.1 BACKGROUND

Mantle cell lymphoma (MCL), which is the topic of this thesis, is a B cell lymphoma. B cells are lymphocytes that are part of our adaptive immune system. Vertebrates have two main defence systems to protect our body against microbes, parasites and viruses; the innate and the adaptive immune system. The adaptive immune system consists of B cells and T cells and eliminates and/or stop the growth of viruses and bacteria that contain non-self-antigens and also creates a long-lived immunological memory that can be activated again, generating a quick response if the antigen is reintroduced. Our innate immune system is composed of several different immune cells such as macrophages, dendritic cells and granulocytes that via Toll-like receptors, phagocytosis and complement cascade try to eliminate an antigen if present for instance in a wound, as a foreign body or invading bacteria. The innate immune system does not have a long-term immunological memory, but it activates the adaptive immune system by presenting non-self-peptides to T and B cells. This can be done by antigen presenting cells (APC) that bind the antigen on HLA molecules, recognized by cells in the adaptive immune system that become activated [1]. B cells are derived from lymphoid progenitor cells that in turn are developed from hematopoietic stem cells. The lymphoid progenitor cell resides in our bone marrow. Via several transcription factors (EBF1, E2A, PAX5) the progenitor cell transits to a precursor and finally to a mature B cell. During this process the B cell needs to develop a unique and functioning B cell receptor (BCR) [2, 3]. Figure 1 shows the BCR that is a transmembrane receptor which on the extracellular site binds antigen and then transmits signals for B cell activation.



**Figure 1.** Schematic picture of BCR with two heavy chains and two light chains and their constant and variable regions on a B cell.

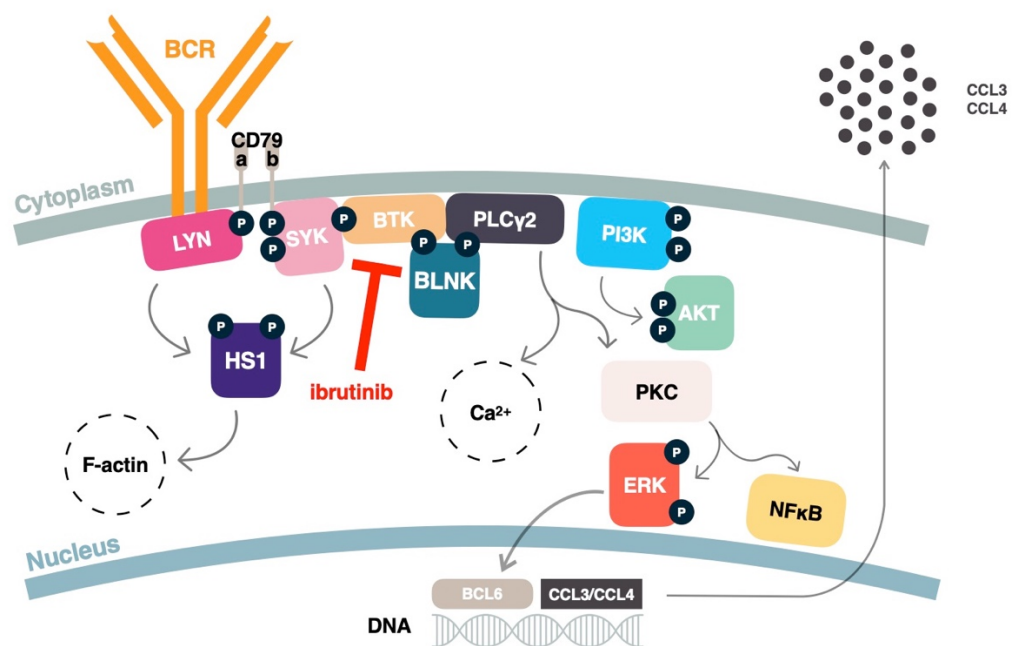
In MCL, BCR signalling is perturbed leading to the constitutive proliferation of the malignant cells [4]. The BCR is composed of an immunoglobulin molecule and several signalling molecules. The immunoglobulin can also be secreted by mature B cells and plasma cells. The immunoglobulin consists of two identical heavy chains and two identical light chains. The heavy chain and the light chain both have constant and variable regions, thus the number of different antibodies that can bind to different antigens are in theory innumerable. The heavy chain is formed with a first step by rearranging the antigen receptor genes at the D heavy ( $D_H$ ) and J heavy ( $J_H$ ) loci resulting in a  $D_HJ_H$  joint and the cell is then called pro-B cell. Next step is for a V heavy ( $V_H$ ) locus to join the  $D_HJ_H$ , resulting in an IG heavy chain that is paired to a temporary light chain. The BCR is now tested for its functional use. If the BCR is ok, then the light chain starts to form by rearranging  $V_L$  and  $J_L$  loci and is expressed as either  $\kappa$  or  $\lambda$ . Now we have an immature B cell [5-7]. To continue to become a mature naïve B cell, the BCR must not show any responsiveness to autoantigens. If so the BCR can undergo additional rearrangements of the light chain or the B cell is driven to an anergic state or apoptosis [3, 8]. The immature B cells leave the bone marrow (BM) and migrates to the spleen where they continue development and become naïve, follicular or marginal zone B cells. The marginal zone cells are our first line of defence to antigens circulating in the blood and when encountering an antigen, the marginal zone cell develops to extrafollicular plasma cells producing and secreting immunoglobulin (Ig) M. This type of response is known as a T cell-independent response [9]. In contrast, the T cell-dependent response takes place in germinal centres of the spleen and lymph nodes where the B cells develop to plasma cells or memory B cells (reviewed in) [10]. The memory B cells are able to survive in niches in the bone marrow for decades [11]. The interaction between B cells and T cells involves numerous receptors and signals, e.g. cluster of differentiation (CD)40-CD40-ligand, interleukin (IL)-21, IL-6 and inducible T cell co-stimulator (ICOS) and ICOS ligand (reviewed in) [12]. The B cells in the germinal centres also interact with follicular dendritic cells and form a light zone where the B cells (centrocytes) are loosely connected and expands, thus squeezing the residing B cells (centroblasts) to a compact, dark zone where B cells are rapidly proliferating and positioned densely. In the dark zone somatic hypermutations (SMH) can occur. SHM can be deletions, point mutations, duplications or insertions in the  $V_H$  and may cause change in the BCR receptor affinity. This can be repeated several times towards getting the best BCR affinity possible for the specific antigen. In the mantle zone, which lays around the dark and light zones, but inside of the marginal zone of germinal centres, small and resting naïve B cells reside [10, 13, 14]. The centrocytes (B cells in the light zone of the germinal centre) can also undergo class switch. Their heavy chains constant region ( $C_H$ ) can be changed through class switch recombination. This results in deletion and replacements of genes coding for the constant region but no changes in the variable region. Thus, IgM and IgD isotypes can be switched to IgG, IgE or IgA. This will keep the affinity for the antigen through the variable region but will change the function of the antibodies [15, 16]. The B cells undergo further differentiation to plasma cells which secrete the antibody that through this whole process has been shown to have the best affinity for the antigen. The long-lived plasma cells reside in specific niches in the BM. Other B cells may differentiate towards memory B cells that leave the germinal centre and can be activated again for a quicker secondary response [17-19]. Summarizing picture is shown in Figure 2.



**Figure 2.** Normal B cell development and different B cell lymphomas. (Modified from Kuppers, R., Mechanisms of B cell lymphoma pathogenesis. Nat Rev Cancer, 2005)

## 1.2 BCR SIGNALLING

The BCR is a transmembrane receptor on the surface of B lymphocytes. It consists of Igs and several accessory signal proteins for example CD79a and CD79b. Activation of the BCR upon binding to an antigen regulates, via downstream signalling, proliferation, metabolism, cytoskeleton remodelling, DNA repair and survival of the B cell [20]. This is executed via signalling cascades in the B cells membrane-proximal kinases e.g. Spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK) and PI3K shown in Figure 3. These kinases also play a role in B cell migration and adhesion [21-23]. Downstream of these kinases, activation of e.g. Ras, mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and other signalling proteins leads to regulation of the B cell's survival [24]. Certain proteins in BCR signalling machinery are also targets for therapy e.g. the BTK inhibitor ibrutinib, successfully used in chronic lymphocytic leukaemia (CLL) and also in MCL [21, 25]. A summarizing picture of BCR signalling is shown below.



**Figure 3.** BCR signalling pathways and the therapeutic agent ibrutinib blocking BTK.



## 2 CANNABINOID RECEPTORS AND ENDOCANNABINOIDS

The hemp plant, *Cannabis Sativa* contains more than 540 different chemical compounds and of those over 100 different cannabinoids.  $\Delta^9$ -tetrahydrocannabinol (THC) is the main biological component (reviewed in) [26]. Several endogenous cannabinoids have been discovered, the first to be described was an eicosanoid derivate, N-arachidonoyl ethanolamine, also called anandamide (AEA) [27]. Others are e.g. 2-arachidonoyl glyceryl (2-AG) [28, 29]. The endocannabinoids are synthesized from cell membrane lipids when needed. Cells synthesizing endocannabinoids are e.g. lymphoid cells in peripheral blood, activated macrophages, microglia, tonsillar B cells, thrombocytes and sensory nerve fibres (reviewed in) [30]. Cannabinoids bind to the cannabinoid receptors, transmembrane G-protein coupled receptors. The two most studied cannabinoid receptors are cannabinoid receptor type 1 and type 2 (CB1 and CB2). CB1 was cloned from brain cells in 1990 and in 1992 CB2 was cloned from an acute myeloid leukaemia (AML) cell line [31, 32]. The gene for CB1 is *CNR1* located on chromosome 6 and CB2 is encoded by *CNR2* located at chromosome 1. In non-malignant tissue CB1 is mostly expressed in neural cells and CB2 in immune cells. Mouse studies have shown that CB2 affects the release of immature B cells from the bone marrow and also plays a role for positioning of B cells in the spleen [33, 34].

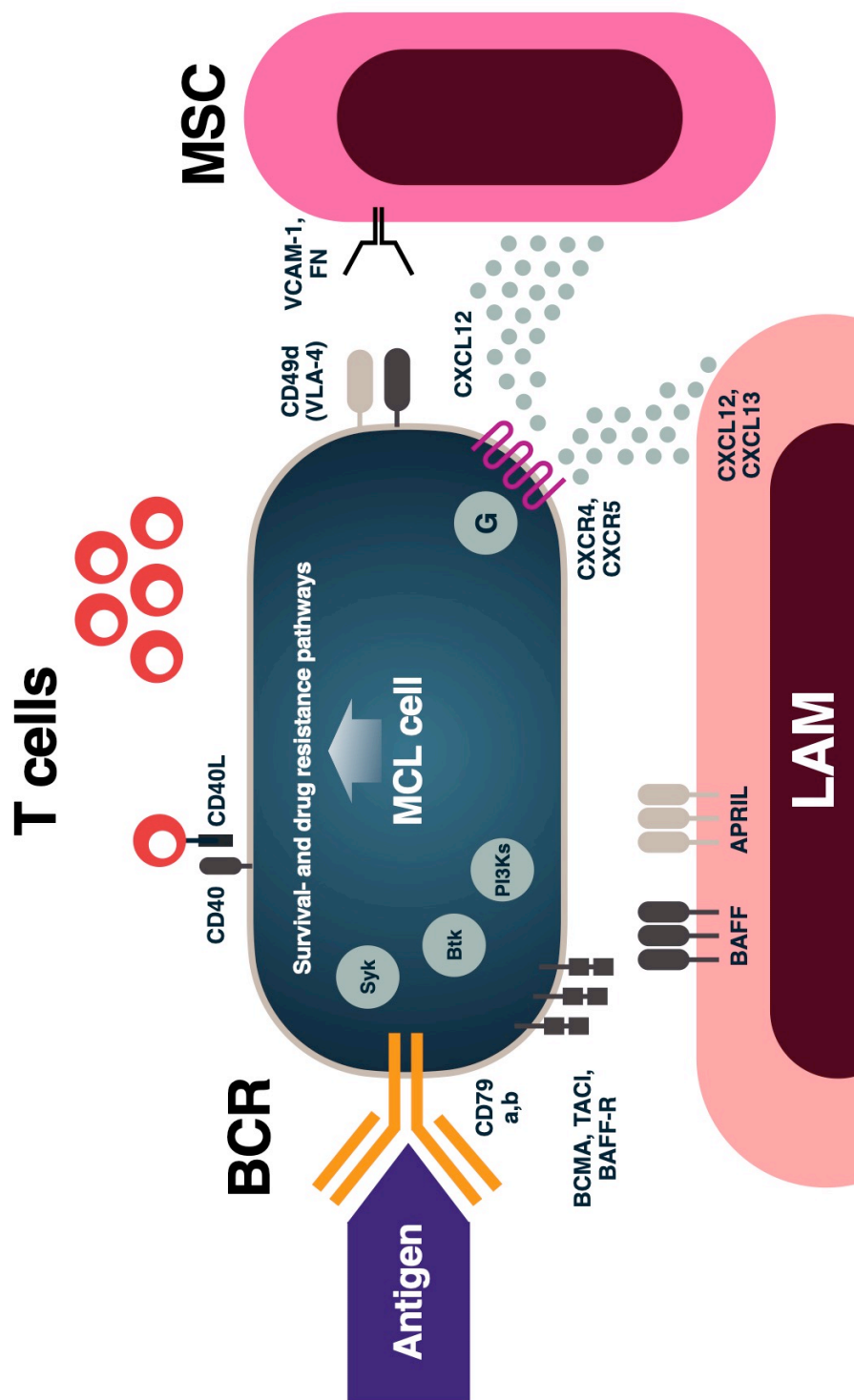
We and others have through gene expression profiling and PCR analysis of messenger ribonucleic acid (mRNA) shown that CB1 and CB2 are more highly expressed in MCL than in purified B cells or reactive lymphoid tissue [35-37]. Our group has also shown that synthetic cannabinoids can induce cell death in MCL *in vitro* and tumour growth reduction in xenograft mouse models [37, 38].

### 3 LYMPHOMAS, SUB CLASSIFICATION, CELL OF ORIGIN CONCEPT AND PATHOGENESIS

Lymphomas are derived from T cells or B cells and B cell lymphomas are more common than T cell lymphomas. Lymphomas can be further divided into Hodgkin lymphoma (a B cell lymphoma) and non-Hodgkin lymphomas (NHL) (most B cell lymphomas and all T cell lymphomas) [39]. NHL constitutes ca 90% of all lymphomas and represent about 4 % of all malignancies in the US [40]. MCL counts for 4% of all lymphomas in the US and 7-9% in Europe [41]. In Sweden we have an incidence of NHL of 21.6 men and 15.3 women per 100 000 persons and year [42].

Lymphomas are often described as aggressive (rapid growth and spread with severe symptoms) or indolent (slow growth and spread with fewer symptoms) e.g. Burkitt lymphoma is an aggressive type and mucosa-associated lymphoid tissue (MALT) lymphoma is considered indolent [40]. Some lymphomas, such as MCL, can present with either an indolent or aggressive clinical course. Lymphomas can be further classified based on their resemblance to normal B cells, the so-called cell-of-origin classification, which is also the basis of the WHO classification of lymphoid malignancies. In this classification MCL bears resemblance to naïve B cells located in the mantle zone of B cell follicles.

The majority of the B cell lymphomas resemble B lymphocytes in the germinal centre or later stages. This has been shown by examining e. g. B cell markers, gene expression patterns comparing lymphomas with purified normal B cell populations, somatic hypermutations and class switch recombination in the malignant B cell. It can be assumed that somatic hypermutation and class switch in the germinal centre generates errors in the DNA which leads to lymphoma development [17, 43]. These errors are usually eliminated, or the cell is designated for apoptosis thanks to several checkpoints. To reach full malignant status a B cell needs to fail in several normal security check points that normally controls proliferation and apoptosis. Many B cell lymphomas also have chromosomal translocations that dysregulates proto oncogenes. These translocations occur during V(D)J recombination and class switch [43]. This is the case in MCL where the cyclin D1 gene (*CCND1*) is translocated to the immunoglobulin heavy chain enhancer. Other examples of genes that are translocated in B cell lymphomas are *BCL2*, *MYC* and *BCL6*, [44-47]. However, in spite of translocations and gene mutations most lymphoma cells cannot survive without interaction with cells in the tissue microenvironment. Both CLL and MCL are lymphomas that mostly proliferate in BM and peripheral secondary lymphoid organs (SLO). In SLO they interact with CD4+ T cells, lymphoma associated macrophages and mesenchymal stroma cells for example as shown in Figure 4 [48, 49]. Furthermore, also viruses can be involved in lymphoma development. For example, Epstein-Barr virus has been connected to development of Burkitt lymphoma, DLBCL, Hodgkin lymphoma and posttransplant lymphoma (PTLD) [50-52]. Human herpesvirus 8 and hepatitis C virus can also drive lymphomagenesis [53, 54].



**Figure 4.** MCL cell interacts with T cells, BCR, mesenchymal stroma cells (MSC) and lymphoma associated macrophages (LAM). (Modified from Burger JA., Ford, RJ., Semin Cancer Biol. 2011)

## 4 MANTLE CELL LYMPHOMA

### 4.1 BACKGROUND

When I first came in contact with MCL in 2003 it had been known as a specific entity for only 9 years. In 1994 it was defined as MCL [55, 56] but already in 1992 it was suggested that the earlier named intermediate lymphocytic lymphoma, mantle zone lymphoma, lymphocytic lymphoma of intermediate differentiation and centrocytic lymphoma was one entity [57]. When the gene translocation t(11;14) was discovered, the proposal for the new entity and the new name MCL instead of centrocytic lymphoma came. The name mantle cell lymphoma refers to their morphologic resemblance to mantle zone B cells (non-neoplastic counterpart), residing in the mantle zone of the follicle [58].

### 4.2 DISEASE DEVELOPMENT

MCL is a B-cell lymphoma constituting 5-10% of all lymphomas [39]. The first event in the pathogenesis of MCL is thought to be the gene translocation t(11;14) (q13;q33) which juxtaposes the gene *CCND1*, encoding the cell cycle regulator cyclin D1 to the immunoglobulin heavy chain (*IGH*) enhancer. This leads to overexpression of the protein cyclin D1. In some instances, the mRNA for cyclin D1 is truncated and lacks sequences that mediate mRNA turnover. This results in a mRNA that has a longer half time than normal one and elevated cyclin D1 protein to drive the cell cycle [59]. There is a subgroup of MCL that lack the t(11;14) translocation and cyclin D1 expression, but then they often instead express cyclin D2 or cyclin D3 [60, 61]. The mutational spectrum and gene expression profiles are similar in cyclin D1 positive and cyclin D1 negative MCL and these are therefore considered to be the same disease [62].

For MCL to develop, additional oncogenic events than the t(11;14) translocation are needed. The t(11;14) translocation occurs in pre-B cells in the BM but MCL is a mature B-cell lymphoma which suggests that the additional oncogenic events take place later in the B-cell development [63]. Those additional oncogenic aberrations have been shown to have impact on DNA damage repair, cell cycle control and cell survival pathways, e.g. p53, CDK4, BCL2 and RB1 [64-66].

Earlier it was believed that MCL arise from naïve B cells, but studies have shown that over 40% of the MCLs have mutated Ig genes and others have Ig receptors that are biased and not randomly selected [67]. This indicates that the tumour cells might have been stimulated by antigen during the development of the disease [68]. The current hypothesis is that MCL arises from a pre-malignant condition where the cells have the t(11;14) but not the additional genetic lesions present in a fully developed lymphoma.

Low levels of peripheral blood lymphocytes with the t(11;14) translocation have been identified in healthy patients as a sign of a premalignant condition. Similarly, few cyclin D1 positive cells can occasionally be detected in the mantle zones of reactive lymphatic tissue (MCL in situ). The t(11;14) translocation is also, together with sex-determining region Y-box (SOX)11 positivity (discussed below), markers for finding minimal residual disease which could lead to relapse of the disease [69]. Interestingly a B cell subpopulation in tonsils believed to be the true counterpart to MCL has recently been identified [70]. In our cohort of

MCL patients we found 10 patients (5,9%) that had sole engagement of MCL in their tonsil at the time of diagnosis. There is also apprehension of subclinical engagement of MCL in this tissue and in the GI-tract, which might be the tissue of location for MCL origination and development [71]. Studies have shown that up to 30% of the MCL patients have symptoms from the GI-tract but when analysing tissue samples from macroscopically normal mucosa in MCL patients with or without gastro intestinal (GI) symptoms up to 80% of the patients have MCL engagement there[72-74]. Since GI engagement is not crucial for decisions regarding therapy it is not a standard procedure to investigate GI tract for possible MCL involvement. MCL have histologically three different growth patterns. MZ pattern, nodular (N) pattern and diffuse (D) pattern, which may carry information on the biology of the disease. The growth patterns have limited prognostic value as a single factor, further discussed below [75].

### **4.3 DIAGNOSTIC PHENOTYPES**

MCL is routinely diagnosed using morphology, immunohistochemistry (IHC) on paraffin embedded tissue sections of e.g. lymph node or other lymphoid tissue and flow cytometry on cell suspensions from lymphoid tissue or bone marrow aspirates. Also fluorescent in situ hybridization (FISH) is used to detect the gene translocation t(11;14)(q13;q33). The most common immune phenotype is CD19+, CD20+, CD5+, CD22+, CD24+, CD79a+, CD43+, FMC7+ and BCL2+ and CD10-, CD11c-, CD23-, CD200- and BCL6- together with cyclin D1+ and SOX11+. However, variations exist: there are about 10% cyclin D1 negative (as mentioned earlier), 5% CD5 negative and 5-10% SOX11 negative MCL cases [76, 77], (reviewed in) [78].

### **4.4 CLINICAL PRESENTATION**

In Sweden approximately 100 new MCL cases per year are diagnosed and the median age at diagnosis is around 70 years but, in some parts of the world e.g. the US as low as 60 years [39, 79]. The larger portion are males and Caucasian. Increased risk of developing MCL is associated to ever living on a farm, having a family history of non-Hodgkin lymphoma or leukaemia and reduced risk is associated to having hay fever [80]. This indicates that MCL could be driven from environmental factors. However, risk factors for developing MCL are not alcohol or tobacco smoking as for most cancers.

MCL is often widely spread at the time of diagnosis and found in BM, blood and extra nodal sites such as the spleen, Waldeyers ring and in the GI tract [81]. At the time of diagnosis, patients often have B-symptoms (fever, >10% weight loss for 6 months, night sweats, fatigue), palpable lymph nodes and sometimes splenomegaly [82]. Blood tests show in most cases as in our cohort (paper IV) no leukemic disease but when analysed by flow cytometry a few MCL cells in the peripheral blood can be detected [83].

## 4.5 INDOLENT MCL

There is a subpopulation of MCL patients that show a more indolent disease course and they do not require any therapy at the time of diagnosis, usually treatment can wait up to several years [84]. In the US deferred therapy for more than 90 days is considered to reflect an indolent disease course whereas in Sweden and other European countries deferred therapy for at least two years has been used to define indolent cases [85]. The decision to start treatment in the indolent patients of our cohort has been based on clinical parameters such as B-symptoms, high lymphocytosis or large tumour burden or large infiltration in the BM. However, it is difficult to identify these patients at diagnosis, which implies risk for overtreatment. In order to find markers for these indolent cases it is important to investigate the pathogenesis and course of MCL. Lately it has been found that a combination of leukemic, non-nodal (or very little nodal engagement) disease with splenomegaly and bone marrow engagement of MCL in the patients predicts a more indolent course [39, 86]. Summarization of the usual characteristics of the classic (nodal) and non-nodal MCL is shown in Table 1.

<b>Classic (nodal) MCL</b>	<b>Non-nodal MCL</b>
unmutated IGHV-regions	mutated IGHV-regions
SOX11-positive	SOX11-negative
involves lymph nodes	non-nodal disease
involves extra nodal sites e.g. GI-tract	involves peripheral blood, bone marrow and spleen
aggressive disease	indolent disease

**Table 1.** Characteristics of classic and non-nodal MCL as defined in WHO classification of tumours of haematopoietic and lymphoid tissues, 2016.

## 4.6 THE ROLE OF THE MICROENVIRONMENT IN MCL PATHOGENESIS

The surrounding cells of the MCL also seem to have importance for survival and evolvement of the lymphoma. This has been shown using CD40/ CD40 ligand (CD154) stimulation in which T cells interact with and affect the MCL cells [87]. Furthermore, a large number of macrophages are found in the microenvironment surrounding the MCL. It has been found that they correspond to more aggressive, high mitotic MCL disease [88]. Pro-proliferative signals increase the risk for further genetic aberrations in addition to t(11;14). Antigen stimulation could thus lead to transformation from indolent to aggressive MCL. This indicates that the microenvironment has impact on the development of MCL [89, 90].

In follicular lymphoma, patient survival has been found to be associated to molecular features of non-malignant cells in the tumour microenvironment [91-93].

The microenvironment in the tissue of MCL contains various non-malignant cells such as T cells, macrophages and stromal cells which are important for growth stimulation and prevention of apoptosis of the MCL cells, thus the lymphoma cells are not autonomous, earlier shown in Figure 4. This was shown by Medina et al. where *ex vivo* isolated MCL cells kept in cell culture without any supportive cells had much shorter survival compared to MCL cells co-cultured with stromal cells, 2 weeks versus at least 7 months, respectively [94].

## 4.7 CLINICAL AND BIOLOGICAL PROGNOSTIC MARKERS

Prognostic markers for MCL are desirable and some markers are discussed below. Among clinical prognostic markers the Mantle cell lymphoma International Prognostic Index (MIPI) is widely used [95]. MIPI includes age, lactate dehydrogenase (LDH), lymphocyte count and performance status according to Eastern Cooperative Oncology Group (ECOG). MIPI has been a controversial tool, but studies have shown good validity also with today's developed therapies including monoclonal antibodies [96]. Lately it has shown to be an even more precise tool when adding information about the proliferation, defined as a cut-off 30% IHC-stained Ki-67 lymphoma cells, known as MIPI-c (combined). This resulted in four different risk groups with a range of 5-year overall survival (OS) from 17% to 85% [75].

The tumour cell morphology may be associated to prognosis. In MCL there are several morphologic variants, such as a small cell variant, classic centrocytic variant and blastoid variant (BV). BV has a homogenous population of cells with blastoid or pleomorphic morphology, high number of genetic aberrations, high proliferation, often *TP53* mutations and an aggressive clinical course [97, 98]. Also, the growth pattern of MCL has been discussed as prognostically relevant and is divided into three types, MZ, N and D growth pattern. These growth patterns showed to correlate to early or late stage of the disease and to be associated to survival [82, 99], though in later studies it has not on its own been shown to have prognostic value [75]. We analysed the growth patterns in our paper III but could not find statistically significant correlation to overall survival (OS).

Genes that affect cell cycle control and transcription have been reported to carry prognostic information i.e. high expression of *CCND1*, high expression of *MYC* or *MYC* translocations, *TP53* mutation or inactivation and high proliferation are all negative prognostic markers [62, 100, 101]. *TP53*, a tumour suppressor gene often mutated in MCL, has been shown to be

associated to a more aggressive type of MCL and is an independent negative prognostic marker for the disease also in patients undergoing ASCT [102-107]. Mutations of the *TP53* gene often increase the expression of the protein p53 since the mutated protein is more stable and accumulate in the nucleus [108]. There are also novel tumour markers suggested to be associated to prognosis. For example, low absolute natural killer cell count in the peripheral blood in MCL patients has shown to be associated to inferior OS [109]. Also, a high degree of overlap of follicular dendritic cell meshwork and tumour area showed better prognosis [110].

#### **4.8 SOX11**

The first SOX protein discovered was SRY (sex determining region on the Y chromosome) in 1990 and was the gene responsible for male differentiation on the Y chromosome ultimately differentiating Sertoli cells and testis development [111]. SRY proteins are only found in mammals and play a large role in cell differentiation [112]. In humans there are 20 SOX proteins found [113]. They are a group of transcription factors that bind to DNA segments and are expressed during foetal life and involved in organogenesis [114].

Normal lymphocytes lack SOX11 expression while MCL highly expresses SOX11 in most of the cases (>90%) [115]. SOX11 has been shown to be involved in cell differentiation and neurogenesis in the central nervous system [116].

A previous study from our group, including 53 cases, showed that MCL patients with SOX11-negative tumours had shorter OS, which could indicate that SOX11 is of prognostic importance [117]. This study was small and later came contradicting studies from other groups indicating that SOX11-negative tumours were indolent [118]. Both the first study from our group and the later study from another group were based on a selected patient material, which is why we chose to investigate SOX11 in our unselected population-based cohort from the Stockholm area in paper I. After this, others confirmed our results [119] and it is now believed that SOX11 cannot predict outcome alone. However, there is a clinical pattern in some MCL patients with the non-nodal MCL described above that are usually SOX11-negative with a more indolent disease course [39, 119].

#### **4.9 THE CHOICE OF THERAPY**

Conventional MCL therapy in patients below 70 years of age at diagnosis and with no comorbidities consists of chemotherapy e.g. cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) with alternating cycles of cytarabine, both in combination with the monoclonal antibody rituximab (R) [120, 121]. This protocol called the Nordic protocol MCL2 was developed by the Nordic Lymphoma Group and several studies on this have been published [122-125]. This intense treatment combined with autologous stem cell transplantation (ASCT) has increased the median overall survival to close to 13 years [125]. Patients over the age of 70 mostly receive R-CHOP with rituximab maintenance or R-bendamustine. When the disease is limited (Stage I and II with one to three adjacent lymph node territories) it is usual to treat this with only radiotherapy to the affected area [120]. Relapse therapy and therapy in chemotherapy refractive patients can be the above



mentioned in different combinations with the BTK-inhibitor Ibrutinib [126], (reviewed in) [127]. In patients with indolent disease presentation a watchful waiting approach is also sometimes practiced [128].

## 5 OVERALL AIM

The aim of my thesis is to identify clinical and/or pathological markers that can predict outcome or serve as new potential targets for therapy in MCL in our population-based cohort. The studies include investigations of p53, SOX11, the effect of upregulated cannabinoid receptors, tumour growth patterns and proliferation and clinical variables such as stage and leukemic disease. Further we here investigate the importance of non-malignant cells in the microenvironment and their correlation to tumour features and prognosis.

### 5.1 SPECIFIC AIMS

**Paper I:** To investigate if SOX11 can be used as a marker to identify aggressive or indolent disease in a population-based cohort.

**Paper II:** To investigate the correlation of upregulated CB1, CB2 and the enzymes regulating the endocannabinoid anandamide in MCL to biological features of the disease.

**Paper III:** To analyse the role of T cells in MCL patients as for tumour growth patterns and predict outcome.

**Paper IV:** To analyse the clinical course, comorbidities, types of treatment and outcome of all MCL patients diagnosed in the Stockholm region during 1998-2013.

## 6 MATERIAL AND METHODS

We retrieved all patients diagnosed with MCL according to the World Health Organization (WHO) classification from 1 of January 1998 to 31 of December 2013 at the Pathology Department, Karolinska University Hospital and S.t Görans Hospital, Stockholm. One experienced pathologist other than the one diagnosing reviewed all cases and verified the diagnosis. Clinical data was gathered from hospital charts. Flow cytometry analysis was performed as a diagnostic routine on lymph nodes and immunohistochemistry (IHC) staining was performed on paraffin-embedded diagnostic biopsies such as lymph node, BM, tonsil, spleen and extra nodal sites. RNA isolation, complementary deoxyribonucleic acid (cDNA) synthesis and quantitative polymerase chain reaction (qPCR) was performed as described in paper II. OS was calculated from time of diagnosis until time of death or last follow up and lymphoma specific survival (LSS) from time of diagnosis until time of MCL-associated death or last follow up. OS were calculated with Kaplan-Meier curves and log rank test. For comparison between groups we used t-test, multivariate analysis, Spearman test, Fishers exact test, Mann-Whitney-Wilcoxon test and logistic regression where needed. P-value < .05 was considered statistically significant. The analysis was made by a statistician in the first article and in the second and third by co-authors and the fourth by me with help from co-authors using Stata and Origin 8.

## 7 RESULTS AND DISCUSSION

### 7.1 Paper I

#### **Prognostic role of SOX11 in a population-based cohort of mantle cell lymphoma**

The material comprised 186 MCL patients, diagnosed in the Stockholm area from January 1, 1998 to June 30, 2010. The cases were retrieved through searching the pathology database Sympathy Pathology and the Regional Cancer Centre Registry. Clinical and pathological data was collected from charts and in paraffin embedded tumour tissue expression of p53, SOX11 and the proliferation marker ki-67 was investigated by IHC.

The cohort was studied with respect to indolent versus aggressive disease course by defining indolent as not requiring treatment during the first two years after diagnosis. 166 patients had aggressive and 17 had indolent disease, respectively. As expected, we found a statistically significant difference in presence of B-symptoms, proliferation (Ki-67>30%) and OS between the two groups. The median LSS time was not reached for the indolent MCL and was 3.1 year (1133 days) for the non-indolent MCL. As for earlier known prognostic markers such as CD23 expression on tumour cells, immunohistochemical p53 positivity and blastoid MCL we did not find any statistically significant difference between the indolent and non-indolent groups. Of the indolent cases 15 out of 17 (88%) were SOX11 positive as well as 142 out of 153 (93%) of the aggressive MCL. We also compared the SOX11 positive (n=160) and SOX11 negative (n=13) cases. Using univariate analysis, we found that expression of SOX11 was associated with longer OS and lack of SOX11 with shorter OS. Interestingly most SOX11 negative tumours expressed high levels of p53 protein, indicating mutated *TP53*. In multivariate analysis SOX11 did not remain a prognostic marker but p53, high LDH, ECOG>2 and age>65 did. In conclusion SOX11 cannot be used as a single marker for predicting indolent or aggressive disease course in MCL.

## 7.2 Paper II

### Perturbations of the endocannabinoid system in mantle cell lymphoma

In this study we analysed 107 samples of MCL; 100 from the time of diagnosis and 7 relapses. Tissue samples of MCL tumour from lymph node (n=81), tonsil, spleen, GI-biopsies and cell suspensions of blood, BM and pleural fluid was used. As described earlier by our research group, the two cannabinoid receptors CB1 and CB2 are upregulated in MCL compared to non-malignant lymph nodes and purified non-malignant B cells and that exposure of cannabinoids to MCL induces cell death *in vitro* [35, 38]. The endocannabinoid system (ECS) consists of the endocannabinoids, the two receptors CB1 and CB2 and the enzymes regulating endocannabinoids' levels. One of the major endocannabinoids is anandamide and its main synthesizing and metabolizing enzymes being N-acyl phosphatidylethanolamine phospholipase D (NAPEPLD) and fatty acid amide hydrolase (FAAH) respectively. Function of cannabinoid receptors and endocannabinoids have been reported to have an important role in other cancers e.g. gastric cancer, leukaemia and glioma [129-131], (reviewed in) [132, 133]. Therefore, we analysed the expression of CB1, CB2 and the synthesizing and metabolizing enzymes of anandamide in these 107 MCL patients. To investigate their importance and function we correlated the data to clinical and pathological features. By using qPCR analysis, we found that NAPEPLD was highly expressed in all MCL cases analysed but absent in normal B cells. Further the anandamide metabolizing enzyme FAAH was found to be upregulated only in 13 of the 107 MCL cases and downregulated in 95 (88%) cases. The gene for CB1 was overexpressed in 105 cases (98%) and CB2 was overexpressed in all cases compared to normal B cells. The mRNA levels of CNR1 and CNR2 correlated moderately. The upregulated expression of CB1, CB2 and FAAH was found to have no correlation to the amount of tumour cells of the cases as measured by flow cytometry. High FAAH was statistically significant correlated to anaemia, lymphocytosis, leucocytosis and p53-positivity. We found that low expression of CB1 was correlated to lymphocytosis, leucocytosis and high expression of CB1 was correlated to SOX11 positivity. High CB2 expression was only correlated to anaemia. Survival analysis did not show any significant difference with respect to CNR1, CNR2 nor FAAH expression.

These results show that MCL cells would have an accumulation of the endocannabinoid anandamide. The low CB1 expression correlating to lymphocytosis could be explained by CB1 having effects on lymphocyte homing to lymphoid tissue. This is interesting since the MCL cells then lose their pro-survival signals from surrounding cells in the microenvironment of lymphatic tissue or bone marrow. Clinical studies of cancer treatment with ligands to CB1 and CB2 have been done [134].

### 7.3 Paper III

#### T-cell levels are prognostic in mantle cell lymphoma

We hypothesized that MCL might be dependent on non-malignant cells in the tissue microenvironment. In order to study this further, we analysed the frequency of non-malignant T-cell and B-cells in diagnostic MCL lymph nodes by flow cytometry and related the findings to growth patterns, cell proliferation and clinical outcome. Flow cytometry data from all diagnostic lymph nodes (n=154) in our extended MCL cohort (including 244 patients diagnosed with MCL in the Stockholm region from January 1, 1998 to December 31, 2012) was evaluated. We analysed frequencies of tumour cells (based on MCL phenotype and expression of Ig light chains as a clonality marker), remaining normal B cells, CD3+ T cells and CD4/CD8 ratio. Analysis of CD56+ natural killer (NK) cells and CD14+ monocytes/macrophages were done only in a subset of the diagnostic flow cytometry analyses and were therefore not included in the final analysis. Cell frequencies was expressed as percentage of cells in the mononuclear gate.

Indolent disease was defined as above, not requiring treatment during the first two years after diagnosis. Higher CD4/CD8 ratio as a continuous variable was positively correlated to indolent disease and longer OS and negatively correlated to high tumour cell proliferation and higher p53 expression by IHC. Data are shown in Table 1 and 2 in paper III. The different growth patterns i.e. MZ, N and D growth pattern correlated to CD4/CD8 ratio and T cell percentage (Figure 1 and 2 in paper III) but not to OS. When analysing MCL grouped by growth pattern we found that indolent disease was statistically significant more common in MZ compared to D. In line with this, D had significantly more often elevated LDH, high proliferation and lymphocytosis than MZ. Further analysis also showed a statistically significant decrease in T cell frequencies and CD4/CD8 ratio during development from MZ growth pattern to D, mainly due to a reduction of CD4+ T cells (Table 2 in paper III). Comparison with reactive lymph nodes (n=26) showed that T cell frequencies in MZ were more similar to non-malignant lymphoid tissue (Figure 2 in paper III). In conclusion we have shown that the microenvironment of the normal lymph node is more preserved in indolent MCL and in MCL with MZ growth pattern. Higher CD4/CD8 ratio was positively correlated to indolent disease and longer OS and negatively correlated to high tumour cell proliferation. Since the CD4/CD8 ratio is independent of tumour burden (amount of tumour cells) and MCL cells earlier has shown to impair T cell responses [135], our results could indicate that MCL gradually progress towards being less dependent on signals from the microenvironment and/or that the immunological tumour control mechanisms are inactivated by lymphoma mediated suppression in aggressive MCL.

## 7.4 Paper IV

### **Mantle cell lymphoma, a population-based long-term follow-up study on patients in the Stockholm region; clinical and pathological presentation, course and treatment.**

We have retrieved 258 patients diagnosed with MCL in the Stockholm area between January 1, 1998 and December 31, 2013 and studied them regarding clinical and pathological parameters, treatment and disease course with long-term median follow-up of 3,9 years for all patients and 8,1 years for surviving patients. The majority of the patients were male (69,4%), older than 65 years (66%) and had stage IV disease (85,8%), SOX11 positivity (91,7%) and non-indolent disease (88,7%) at the time of diagnosis. At the last follow-up 92,2% of the patients had received some kind of treatment, the most frequently being R-CHOP, CHOP, sole per oral alkylating agents, R-bendamustine and radiation therapy. In total, 27,3% of the treated patients were consolidated with ASCT. Median OS for the whole cohort was 3,9 years and for the patients treated with or intended for ASCT (thus receiving the same intense induction therapy) was 16,3 years. Patients before or during 2003, received rituximab more seldom as part of the treatment compared to patients treated after 2004, when rituximab was included in the Swedish treatment guidelines for MCL. Patients receiving rituximab had both statistically significant longer OS and LSS than patients not receiving rituximab as part of their first line treatment (OS 5,3 and 2,5 years, respectively and LSS 8,3 and 3,1 and years, respectively). Among our 258 MCL patients, 69 were diagnosed with another type of cancer before or after their MCL diagnosis, but only one case of therapy related myeloid malignancy and this after MCL treatment. In our cohort 28/254 patients had an indolent disease course, 22 men and 6 women. As expected, the patients with indolent disease had at the time of diagnosis a less bulky tumour burden, fewer symptoms, less frequently blastoid variant and lower LDH levels than the non-indolent patients. Median OS for these patients was 6,9 years and median LSS was 11,5 years. Regarding gender differences, women were older than males at the time of diagnosis. There was no statistically significant difference between men and women as of clinical and pathological markers except for LDH-level that was higher in women. Neither time from diagnosis to treatment start, nor frequency of consolidation with ASCT, OS or LSS was statistically significantly different between the genders and OS and LSS were comparable. The rate of p53-positivity and SOX11-positivity was 17,7% and 91,7% respectively, in line with other research groups results [119]. The treatment regimens and frequency of ASCT was also in concordance with a larger Swedish register study [79]. The monoclonal antibody rituximab often used in MCL therapy has different clearance in men and women. We investigated, but found no difference, in the male/female ratio of patients receiving or not receiving rituximab as part of their first line therapy. Due to their in average bigger body surface area men often receive a larger dose than women [136]. Although OS and LSS was similar in males versus females when analysed separately in those receiving rituximab-containing treatment, suggesting that also females receive an optimal dose of rituximab.

Furthermore, we found male gender to be a positive prognostic factor for OS similar to Cohen et al. [85]. However, in most studies male gender is a negative marker for prognosis [85, 95, 137]. In conclusion we here show real-world data on non-selected population-based MCL patients with male gender being more frequent but surprisingly also a good prognostic factor. We showed that the women are older at the time of diagnosis and that they have higher LDH than men. We also showed that the use of rituximab after its inclusion in the

Swedish treatment guide lines doubled and also increased OS. Whereas we could not see that the non-nodal MCL cases were indolent nor that indolent cases could be identified by SOX11-positivity.



## 8 FUTURE PERSPECTIVES

In further studies we hope to find new prognostic factors that correlate to indolent or aggressive disease. The importance of early knowledge of the disease course is essential in order to better select patients not in need of intense treatment at the time of diagnosis. Also new potential drugs and therapies are welcomed. CB1 and CB2 ligands being one path. Our research group has recently conducted one small clinical trials of clinically approved cannabinoids in leukemic, indolent lymphoma, and the results are currently analysed. Furthermore, chimeric antigen receptor T cell (CAR-T) treatment is already ongoing in other lymphomas targeting for example CD19 (reviewed in) [138]. Development of CAR-T that can target more than one CD is ongoing which would specify and narrow down the treatment even more, avoiding side effects [139]. To be able to combine several CDs in CAR-T could customize therapy in MCL. Another way of helping MCL patients survive is increased knowledge on how to avoid relapses. One could do that by studying how to find minimal residue disease (MRD) in the BM of the MCL treated patients who seem to be in complete remission [140]. These few MCL cells that compose the MRD are difficult to detect with conventional laboratory methods used to diagnose MCL such as immunohistochemistry and flow cytometry and highly sensitive PCR methods are needed. It has been discussed in studies that the most used qPCR analysis of t(11;14)(q13;q32) to find MRD could be inaccurate since other NHL also can have this translocation but that SOX11 might be a more specific marker [141, 142]. But then what about the SOX11-negative MCL cases? It would also be interesting collecting MCL MRD cells, analyse them regarding genetic and epigenetic changes and compare them to the patient's diagnostic phenotype. This could be done with next-generation sequencing (NGS) of DNA. Maybe one might then find the differences that made these MRD cells survive the therapy given and find even other potential new targets of therapy.

Since we found that higher CD4/CD8 T cell ratio was positively associated to indolent disease and longer OS and negatively correlated to high tumour cell proliferation one would also want to investigate which CD4+ T cell subsets there are in the MCL e.g. CD4+FOXP3+ and CD4+PD1+. The CD4+PD1+ T cells have shown to not respond to cytokines in follicular lymphoma and in tonsils [143]. Also, studies of MCL cells showed that the malignant cells *in vitro* efficiently inhibited CD4+ and CD8+ T cell proliferation and T cell-mediated cytotoxicity [135]. This together indicates that T cell signalling, and T cell responses could be important for the survival of the tumour cells. The immunomodulatory agent lenalidomide which has been showing good results in relapsed or refractory MCL through antitumor and antiproliferative effects is very interesting [144]. Lenalidomide can block angiogenesis and tumour cell proliferation and can stimulate T and NK cell-mediated cytotoxicity and increase the amount of NK cells (reviewed in) [145]. We believe that targeting or modifying the microenvironmental cells is one of the keys to better MCL therapy.

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